

The Effects that Non-steroidal Anti-inflammatory Drugs (NSAID) have on Cancer Related  
Fatigue in a Mouse Model

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## Abstract

**Purpose:** Fatigue is a debilitating symptom in cancer patients. It is believed that skeletal muscle wasting is a main cause of cancer related fatigue (CRF). As of now, there is no effective treatment of CRF. According to the 2009-2013 Research Agenda of the Oncology Nursing Society, a high priority in cancer research is symptom management. There is some evidence that nonsteroidal anti-inflammatory drugs (NSAIDs) reduce muscle wasting in tumor-bearing mice. The purpose of this study is to determine the effects of ibuprofen, an NSAID, on fatigue, muscle mass, and biomarkers of muscle protein breakdown in mice bearing the Lewis lung carcinoma. In mice, fatigue is measured as a decrease in voluntary wheel running activity (VMRA) relative to a baseline. **Sample and methods:** The sample is 24 adult, age-matched female C57BI/6 mice. The mice were acclimated to the cages and wheels for 7 days. On day one, half of the mice were inoculated with tumor cells and half served as healthy controls. Half of each group were implanted with a pellet designed to release 5 mg/kg/day Ibuprofen over the course of 21 days of tumor-growth or a placebo pellet. The 4 groups are tumor/placebo pellet, tumor/ibuprofen pellet, no tumor/placebo pellet, no tumor/ibuprofen pellet. VWRA, body weight, food and water intake were measured on days 0, 7, 14, and 19 of tumor growth. Mice were euthanized on day 20 and the gastrocnemius muscle was removed and weighed. **Results:** Ibuprofen had no significant effect on VWRA, muscle mass, or biomarkers of muscle protein degradation. There was a significant negative relationship between spleen size, an indicator of systemic inflammation, and muscle mass (-.53), and a significant positive relationship between spleen size and biomarkers of muscle protein degradation (.6), and between VWRA and muscle mass (.4). These data confirm that skeletal muscle wasting is related to inflammation, and muscle wasting contributes to fatigue. More research is needed to develop effective interventions to reduce muscle wasting in patients with cancer-related fatigue.

## Chapter 1: Statement of the Problem

### Introduction

Cancer and its treatments generate symptoms that are difficult to live with. According to the National Cancer Institute, in 2007 there were over 11 million men and women alive with the history of cancer in the United States (Howlander 2010). According to the 2009-2013 Research Agenda of the Oncology Nursing Society, cancer symptom management is a priority. Quality of life is a paramount concern in the clinical management of cancer patients.

Quality of life is negatively affected by the presence of cancer symptoms. One of the main symptoms with predominant effects on cancer patient's lives is fatigue. Cancer Related Fatigue (CRF) manifests in 60-90% of cancer patients (Hauser 2008). Cancer related fatigue is not relieved by adequate sleep, proper nutrition, or a decrease in stressors. CRF can interfere with physical, psychosocial, economic, and occupational aspects of lives. This incurable fatigue significantly changes the life of a cancer patient. Currently, there is no effective and reliable treatment for CRF.

### Background

"The National Comprehensive Cancer Network, defines cancer-related fatigue as a common, persistent, and subjective sense of tiredness related to cancer or to treatment for cancer that interferes with usual functioning" (Gupta 2007). CRF has been reported as the most difficult symptom to cope with by cancer patients. The impact of CRF on patients is devastating. (Wang 2008). Cancer patients experience fatigue throughout cancer treatment, and long after the treatment is over (Wang 2008). This condition is most prevalent in patients with metastasized cancer or patients that are receiving multimodality or high dose treatment regimens. (Hauser 2008). More aggressive treatment therapies and the increase in cancer survival have caused the prevalence of CRF to increase.

The etiology of CRF is still unknown. There are several hypotheses being researched. Most research is directed towards the multifactorial nature of CRF, referred to as a "web of causation" (Wang 2008). CRF is associated with several other symptoms; however, there are no clear precipitating factors. One predominant explanation of CRF is the proinflammatory cytokine hypothesis. Chronic diseases cause a dysregulation in cytokine production. Elevated serum levels of inflammatory cytokines, namely, interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-a), have been related to persistent fatigue in cancer patients (Wang 2008).

These cytokines are also known to cause skeletal muscle wasting. They are either produced by the tumor growth itself or the host response to the tumor growth. IL-6 and TNF-a are associated with skeletal muscle wasting in cancer (Hitt 2005). TNF-a increases the muscle expression of MAFbx and MuRF1, two biomarkers of muscle protein degradation via the ubiquitin-proteasome pathway (Acharyya 2004). TNF-a also increases the muscle expression of Bnip, a protein used in the autophagy of muscle cells (Hui Xu 2011).

Animal models provide a way to look at the biological mechanisms of muscle wasting, and provide a controlled environment to collect data. Previous studies have shown that tumor bearing mice show higher serum levels of pro-inflammatory cytokines than healthy controls (Argiles 2007). This indicates the presence of inflammation. Mice treated with a non-steroidal anti-inflammatory drug (NSAID) had decreased muscle wasting (Graves 2006) and decreased muscle

expression of MAFbx and receptors for TNF (Hitt, 2005). However, is it unknown if improved muscle mass and decreased inflammation will translate into reduced fatigue. In mice, spleen enlargement is used as an indicator of inflammation. Fatigue is modeled as decreased locomotor activity or decreased voluntary wheel running activity (VWRA).

### Purpose of Study

The purpose of this study is to determine the effects of ibuprofen, an NSAID, on fatigue, muscle mass, spleen size, and muscle expression of MurF1, MAFbx, and Bnip, biomarkers of muscle protein breakdown in female mice bearing the Lewis lung carcinoma. We hypothesize that:

- 1) Ibuprofen will preserve muscle mass in tumor-bearing mice.
- 2) Ibuprofen will reduce spleen size in tumor-bearing mice.
- 3) Muscle mass will be related to VWRA.
- 4) Ibuprofen will reduce expression of biomarkers of protein breakdown in muscle tissue of tumor-bearing mice.

### Significance

CRF is difficult for patients and health care providers to manage. More reliable and consistent treatments are needed to control this devastating experience for cancer patients. Previous studies have shown that ibuprofen decreases muscle wasting in tumor-bearing mice. This experiment will determine if ibuprofen has an effect on VWRA in this animal model of CRF. The data from this study will increase our understanding of CRF and contribute to the development of effective treatments for this devastating symptom in cancer patients.

## Chapter 2: Literature Review

CRF is a debilitating symptom experienced by up to 90% of cancer patients. CRF is universal among all types of cancer and more prevalent in advanced cancer. It is associated with poor quality of life, and reduced ability to perform activities of daily living (Hauser 2008). The physical symptoms interact with the emotional symptoms, affecting the patient's perception of disease and ability to cope (Wang 2008). In a study done by Hauser et. al (2008) on the prevalence, severity and characteristics of CRF, patients reported inability to complete important activities and described fatigue as more distressing than pain, nausea, and vomiting.

Pathophysiology of CRF has not been adequately elucidated. Possible causes of CRF include the cancer itself as well as the treatments and symptoms that are commonly clustered with fatigue. Factors known to occur with or contribute to CRF include pain, emotional distress, sleep disturbance, anemia, nutritional deficiencies, cardiac deconditioning, and co-morbidities. However, the factor that is both necessary and sufficient to induce development of CRF has not yet been identified (Wang 2008). The interaction of etiology and host response creates a complicated chain of possible causalities.

Potential causes are grouped into disease related and treatment related factors. For instance, surgical procedures have been known to induce fatigue. Post-operative fatigue can be related to several factors such as anesthesia, analgesics, decreased ventilation capacity, immobilization, infection, or anxiety. Chemotherapy is also a treatment known to cause fatigue. Common co-existing symptoms of chemotherapy such as anemia, nausea, vomiting, and diarrhea can cause patients to experience CRF. CRF may also be associated with accumulated byproducts of apoptosis that occurs during chemotherapy. Fluctuations in fatigue have been noted throughout the course of chemotherapy. Neurotoxic effects of chemotherapy drugs also present as a potential causative factor. Patients have reported that fatigue is experienced more when they are close to their white blood cell nadir, the time when their white blood cell count is the lowest. Patients report that the fatigue decreases as the white blood cell count improves (Wang 2008). Another cancer treatment that is known to cause fatigue is radiotherapy. The side effects of radiotherapy are all potential precursors to fatigue; they include anemia, diarrhea, weight loss, anorexia, and chronic pain. Biological-response modifiers are known to cause such intense fatigue that treatments must be stopped. Some types cause up to 70% of the recipients to experience intense fatigue. Tumor related factors are potential causes as well. Patients with advanced stages of cancer experience more intense fatigue because the progressing tumor and disease have severe effects on several organ systems (Wang 2008).

There are several hypothesized mechanisms of CRF. An organizing hypothesis is the dysregulation of pro-inflammatory cytokines. Pro-inflammatory cytokines affect normal muscle function. Neurophysiologic changes in skeletal muscles have been noted in previous studies (Wang 2008). Cytokines also affect the metabolic process in muscles, causing them to become weak. Skeletal muscle wasting is induced by pro-inflammatory cytokines. Growing evidence shows the important role that pro-inflammatory cytokines such as IL-6 and TNF $\alpha$  have in tumor-induced muscle wasting (Argiles 2009, Tisdale 2009). TNF- $\alpha$  is produced by the host immune cells in response to the tumor and has a significant role in tumor-induced muscle wasting. IL-6 and TNF $\alpha$  are associated with increased expression of the ubiquitin-proteasome pathway (UPP) of muscle protein degradation and autophagy in skeletal muscle. The ubiquitin-proteasome is one of three proteolytic pathways in muscles. UPP takes part in muscle myofilament break down. Animal and human models of muscle wasting show that the UPP contributes to the degradation

of myofibrillar proteins (Tisdale 2009). Increased activity of the UPP is associated with increased expression of MuRF1 and MAFbx which are two muscle-specific E3 ubiquitin ligases that mark myosin protein for degradation by the proteasomes (Achaeyya 2004). The expression of MuRF1 and MAFbx has been shown to increase in animal models of muscle wasting (Dodson 2011). The cytokines also increase expression of Bnip, a protein that is necessary for the formation of vesicles for autophagic degradation of intracellular proteins (Hui Xu 2011). Structural changes in the muscle fiber make the muscle more susceptible to fatigue during exercise, which is modeled as decreased VWRA in a mouse model. Proinflammatory cytokines also increase expression of cyclo-oxygenase (COX) (Hitt 2005). NSAIDs block the COX-1 and COX-2 activity, and have been shown to reduce muscle wasting, and reduce the expression of MAFbx and receptors for TNF in muscle tissue of tumor-bearing mice. NSAIDs may preserve muscle mass by reducing muscle response to the catabolic effects of TNF- $\alpha$  (Graves 2006).

The multifactorial and multidimensional nature of CRF has hindered the development of CRF methodologies. The relationship between pathophysiology and host susceptibility exposes a complex web of causation. Limitations also arise from the differences in patient disease processes and variation in fatigue measurement techniques (Gupta 2006). The inherent subjectivity of CRF has hindered the development of pre-clinical models (Wang 2008). Animal models have been used to study CRF because it provides a more controlled environment to gather data from. Rodents are nocturnal animals and will normally run 2-3 miles during the night. In mice, decreased VWRA indicates illness-induced fatigue (Skinner 2009).

## Chapter 3: Methods

### Introduction

This study used a quantitative, experimental design with random assignment of adult, age-matched female C57BI/6 mice to one of four treatment groups: tumor, tumor plus ibuprofen, no tumor, no tumor no ibuprofen. The mice were housed in their own cages with food and water available at all times. Lewis lung carcinoma (LLC) cells obtained from American Type Tissue Culture (Manassa, VA, USA) were used to induce tumor growth. Mice were inoculated with tumor cells on day one. Body weight, food and water intake, and VWRA were measured on days 0, 7, 14, and 19 of tumor growth. Mice were euthanized on day 20.

### Procedure

The mice were acclimated to the cages and wheels for 7 days. Running wheels were removed from the cages after the acclimation period. On day one, half of the mice were inoculated with tumor cells and half served as healthy controls. The tumor mice were inoculated subcutaneously between the scapulae with  $5 \times 10^5$  LLC tumor cells in .2ml of saline. The control mice were injected subcutaneously between the scapulae with .2ml of saline. Half of each group was implanted with a pellet designed to release 5 mg/kg/day ibuprofen over the course of 21 days of tumor-growth or a placebo pellet. Body weight, food, and water intake were measured on days 0, 7, 14, and 19 of tumor growth. On days 0, 7, 14, and 19 wheels were put into the cages and VWRA was monitored for 24 hours. Mice were sacrificed on day 20 and weighed. The gastrocnemius muscle and spleen were removed and weighed. The samples were wrapped in foil, frozen in liquid nitrogen, and stored at -80 degrees. Weights of right and left gastrocnemius

muscles were averaged to determine muscle weight. Muscle mass was determined by dividing muscle weight by the body weight. Total RNA was extracted from 100mg of frozen gastroc muscle in TRIzol (Invitrogen, Carlsbad, CA). RNA was treated with DNase 1 (Invitrogen) and reverse transcribed to cDNA using the Iscript cDNA synthesis kit (BioRad). Real time PCR was performed using primer pairs for MAFbx and MuRF1. Bnip was detected using a TaqMan Gene Expression Assay according to manufacturer's instruction.

#### Voluntary Wheel Running Activity

VWRA was measured to indicate the presence of illness related fatigue in mice. Healthy mice run from 2-3 miles a night (Skinner 2009). Running wheels were obtained from Columbus Instruments in Columbus Ohio. Wheel turns were counted by a magnetic switch mounted outside of the cage that is triggered by a magnet imbedded in the front of the wheel. The mechanical switches are connected to a cable that plugs into an input module. The boxes are connected to an external computer programed to record the data at preset intervals. Baseline VWRA was recorded on day 0 for each mouse before tumor induction. VWRA was recorded on days 7, 14, and 19 of tumor growth.

#### Drug Treatment

The Ibuprofen pellets were obtained from Innovative Research of American (Sarasota Florida). They are designed to release the drug slowly at 5mg/kg/day. Half of the tumor mice and half of the control mice received a drug pellet and half received a same-sized placebo pellet. The pellets were placed in the subcutaneous space on the right flank of the mice. The mice were lightly anesthetized with inhaled isofluorane and the fur was shaved and cleaned with 70% ethanol. The pellet was inserted using a sterile trochar needle. The incision was closed with dermaglue. Antibiotic ointment was applied to the incision site.

#### Data Analysis

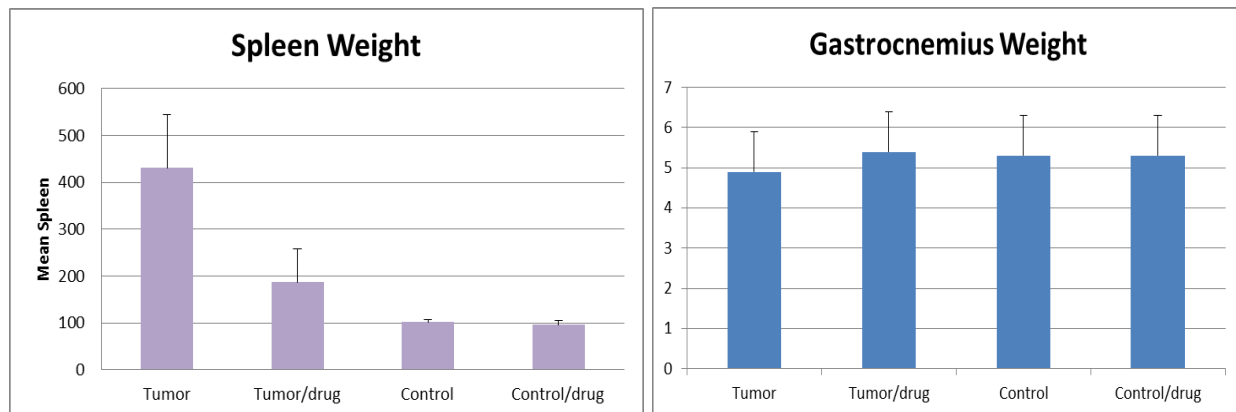
Data was entered into a spread sheet and analyzed using SPSS 16. The independent variables were tumor and drug. The dependent variables were muscle mass, spleen weight, VWRA, and relative expression of Bnip, MAFbx and MURF mRNA in muscle tissue. Data was analyzed using a two way ANOVA to determine main effects of the independent variables on each dependent variable. Bivariate correlations between dependent variables were also examined.

#### Chapter 4:

#### Results

There was no main effect of tumor or drug treatment on muscle mass. Thus, there were no overt signs of cachexia in the tumor-bearing mice.

There was a main effect of tumor on spleen weight ( $p=.000$ ) and a main effect of drug treatment on spleen weight ( $p=.000$ ). Thus, tumor growth induced an inflammatory response, and ibuprofen reduced the inflammation. There was no main effect of tumor or drug treatment on VWRA. Thus, neither tumor growth nor drug treatment affected locomotor activity of the mice. However, there were equipment failures during each session of recording of VWRA. There was a main effect of tumor on muscle expression of MAFbx ( $p=.031$ ), but there was no effect of drug treatment. There was no main effect of tumor or drug treatment on muscle MuRF1. There was a significant tumor effect on muscle expression of Bnip ( $p=.012$ ) and a significant effect of drug treatment on Bnip ( $p=.047$ ). Thus, tumor growth increased expression of MAFbx and Bnip, two biomarkers of muscle protein degradation, but only Bnip was reduced by treatment with ibuprofen.



There was a significant positive correlation between spleen size, and muscle expression of MAFbx ( $r=.72$ ,  $p=.000$ ), MuRF1 ( $r=.56$ ,  $p=.005$ ) and Bnip mRNA ( $r=.61$ ,  $p=.002$ ), supporting the idea that inflammation contributed to muscle protein degradation. There was a correlation between VWRA and muscle mass, suggesting that muscle wasting contributes to fatigue ( $r=.4$ ,  $p=.011$ ). There was a significant negative relationship between spleen size and muscle mass ( $r=-.53$ ,  $p<.001$ ), indicating that the inflammation contributed to smaller muscle mass.

Significant Correlations					
	Spleen	VWRA	MAFbx	MuRF1	Bnip
Spleen	1	-.5	.7	.5	.6
Muscle Mass	-.5	.4			

These data confirm that skeletal muscle wasting is related to inflammation, and muscle wasting is related to fatigue. The reduced spleen size in mice treated with ibuprofen verifies that ibuprofen did reduce inflammation in the tumor-bearing mice, and reduced expression of a biomarker of autophagy, though it did not affect muscle mass directly.

## Chapter 5:

### Summary of Findings



CRF is present in 60-90% of cancer patients and is repeatedly reported as the most debilitating symptom by cancer patients. It reduces quality of life and contributes to cancer morbidity. The multifactorial nature of CRF has made it difficult to discover an effective method of management. Palliative care and symptom management are essential in preserving the quality of life for cancer patients. Currently, there are no reliable treatments of CRF. Previous research has shown that ibuprofen, an NSAID, preserved muscle mass in tumor bearing mice (Graves 2005). This research was performed to explore the effects of ibuprofen on fatigue in tumor bearing mice. Fatigue in mice is modeled as a decrease in VWRA. In the present study, we failed to demonstrate an effect of tumor growth or ibuprofen on the gastrocnemius muscles of the tumor bearing mice nor their VWRA. However, tumor growth and drug treatment showed significant effects on spleen weight. The spleens of the tumor bearing mice were significantly larger than the control group, and were reduced by treatment with ibuprofen. This reduction in spleen size was accompanied by the reduced expression of MAFbx and Bnip in muscles of tumor bearing mice treated with ibuprofen.

## Conclusions

Ibuprofen produced an anti-inflammatory effect indicated by a decrease in spleen weight of the tumor-bearing mice. Ibuprofen also decreased expression of Bnip mRNA in gastrocnemius muscles of tumor-bearing mice. However, ibuprofen had no effect on muscle mass or VWRA of the tumor-bearing mice. It should be noted that expression of MAFbx and Bnip mRNA was increased in muscles of tumor-bearing mice, which suggests that wasting may have occurred had the study duration been longer. We were unable to obtain reliable group measures of VWRA on days 0, 7, and 14, with failure of data collection on day 19. However, muscle mass was correlated with VWRA on day 14, supporting the idea that muscle wasting contributes to CRF. Further research is needed to develop a better understanding of the pathology of CRF and to discover effective interventions to preserve muscle mass and reduce fatigue in cancer patients. Improving the understanding of the nature of CRF can ultimately lead to more effective management regimens. When pathophysiologic mechanisms are better understood, clinical studies should be performed to improve CRF detection and treatment. The only effective way to battle CRF is from a multidisciplinary approach.

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